Detection of an Intragenic Deletion Expands the Spectrum of *CTSC* Mutations in Papillon–Lefèvre Syndrome

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The Papillon–Lefèvre syndrome (PLS) is an autosomal recessive disorder. The gene responsible for the disease, cathepsin C (*CTSC*), is localized in 11q14.1–q14.21. We performed mutational and functional analyses of *CTSC* in two patients affected by this condition. Three previously unreported *CTSC* mutations were identified. The first patient had a compound heterozygous status with a p.G386R missense mutation and an intragenic deletion spanning exons 3–7. Second patient carried a homozygous splice site mutation, p.A253SfsX30. *CTSC* activity was undetectable in both patients, thus demonstrating the pathological effect of these mutations. We describe early evidence of an original intragenic deletion reported in PLS. Since this mutational mechanism could not be detected by direct sequencing, intragenic deletion has to be specifically investigated using gene dosage analysis techniques such as quantitative multiplex fluorescent polymerase chain reaction. We consider that this technique should be performed in patients with apparently homozygous CTSC mutations when one parent does not carry the expected mutation or is not available for analysis.

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INTRODUCTION

Papillon–Lefèvre syndrome (PLS) (OMIM 245000) is a rare autosomal recessive disorder characterized by the association of palmoplantar keratoderma and periodontitis. Cathepsin C (CTSC), a lysosomal cysteine protease, plays an essential role in the activation of serine proteases, particularly leukocyte elastase and granzymes A and B.

We report hereafter the results of genetic investigations obtained in two PLS families. The functional consequences of these mutations were explored using enzymatic assays to confirm their pathological effect.

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Abbreviations: CTSC, cathepsin C; QMF-PCR, quantitative multiplex fluorescent-polymerase chain reaction; PLS, Papillon–Lefèvre syndrome

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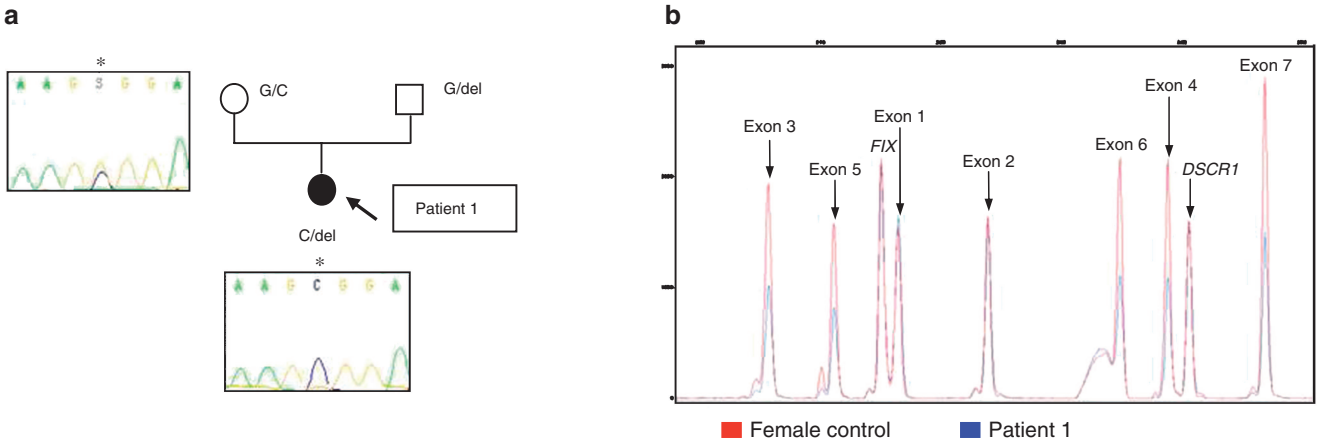
RESULTS

Patient 1 exhibited the most interesting findings with the identification of a compound heterozygous status composed of two novel mutations. Firstly, the gene sequencing of this case displayed a missense mutation p.G386R (Figure 1a). Although this finding was rather suggestive of a homozygous mutation, sequencing of parents' DNA revealed a heterozygous p.G386R mutation in her mother but not in her father. Microsatellite analyses performed in this patient and her parents allowed us to exclude false paternity and uniparental isodisomy (data not shown). We therefore suspected the presence of a concomitant large deletion of *CTSC* transmitted by her father on the second allele. A gene dosage analysis was performed over the entire coding region of *CTSC* by quantitative multiplex fluorescent-polymerase chain reaction (QMF-PCR) technique (Niel *et al.*, 2004). Experimental conditions were optimized for each exon and validated using the DNA of healthy controls and of a deleted control corresponding to a case who carried a previously described 11q14.3 deletion including *CTSC* (Goizet *et al.*, 2004). The QMF-PCR analysis demonstrated the presence of a heterozygous intragenic deletion of *CTSC* involving exons 3–7 both in patient 1 and her father (Figure 1b and c).

In patient 2, we identified a homozygous splice site mutation, c.757G>A, affecting a highly conserved residue. Moreover, this mutation alters the donor splice site at the end of exon 5 with a predictive score *in silico* decreasing from



c

	Height value		Fluorescence ratio	
	Female control	Patient 1	*DSCR1* exon4	*FIX* exon 5
Reference genes				
DSCR1 exon4	1,605	1,587	-	1.02
FIX exon 5	2,164	2,099	0.98	-
CTSC exons				
Exon 1	1,544	1,632	1.07	1.09
Exon 2	1,640	1,571	0.97	0.99
Exon 3	1,944	1,023	0.53	0.54
Exon 4	2,159	1,088	0.51	0.52
Exon 5	1,585	818	0.52	0.53
Exon 6	2,176	1,105	0.51	0.52
Exon 7	2,903	1,501	0.52	0.53

	Height value		Fluorescence ratio	
	Male control	Patient 1's father	*DSCR1* exon4	*FIX* exon 5
Reference genes				
DSCR1 exon4	1,516	1,379	-	1.03
FIX exon 5	1,044	926	0.98	-
CTSC exons				
Exon 1	1,414	1,489	1.16	1.19
Exon 2	1,547	1,368	0.97	1.00
Exon 3	1,738	882	0.56	0.57
Exon 4	1,968	955	0.53	0.55
Exon 5	1,482	725	0.54	0.55
Exon 6	1,932	946	0.54	0.55
Exon 7	2,733	1,315	0.53	0.54

Figure 1. *CTSC* mutation analysis of patient 1. (**a**) Patient 1 carries a heterozygous mutation c.1156G>C (p.G386R) inherited from her mother. Electrophoregrams are shown for patient 1 and her parents. The asterisk shows the location of the base transition. (**b**) Characterization of exons 3–7 deletion inherited by QMF-PCR. The *x*-axis displays the computed length of the PCR products in base pairs as determined using an internal standard (ROX350, Applera). The *y*-axis shows fluorescence intensities in arbitrary units. Gene fragments are indicated above each peak. Patient 1 fluorogram is in blue, while the female control is in red. (**c**) Fluorescence ratios are calculated as indicated in the Materials and Methods section. As an example, the ratio for CTSC exon 4 compared to DSCR1 exon 4 is: patient 1 (CTSC Ex4/DSCR1)/Control (CTSC Ex4/DSCR1) = (1088/1587)/(2159/1605) = 0.51. Gene dosage anomalies are in red. Left: fluorescence ratio calculation for patient 1; right: fluorescence ratio calculation for her father. This demonstrates that the deletion was inherited from the father.

0.98 to 0 (Splice Site Prediction by Neural Network). This suggests the absence of intron 5 splicing. Both asymptomatic parents were shown to be heterozygous for this mutation (Figure 2).

The functional analyses showed an almost undetectable level of CTSC activity in the two patients carrying CTSC mutations confirming their pathological effect (Table 1).

DISCUSSION

More than 50 mutations have been reported so far in PLS. Missense and nonsense mutations are the most frequent, but small deletions, insertions and splice site mutations have also been reported (Selvaraju *et al.*, 2003). The data obtained in patient 1 are highly remarkable. Indeed, this patient showed an unreported compound heterozygous *CTSC* mutation which associates a p.G386R mutation on the maternal allele and an intragenic deletion including exons 3–7 on the paternal allele. The p.G386R mutation affects a glycine residue, which is highly conserved among species and other cysteine proteases including cathepsins. The substitution by a positively charged arginine is therefore probably deleterious. Additionally, the QMF-PCR analyses showed a 50% reduced gene dosage of exons 3–7 of *CTSC* (Figure 1). This intragenic deletion leads to a truncated protein containing only the first two exons of the gene.

Patient 2 presented a previously unreported homozygous c.757G>A mutation, p.A253SfsX30, inherited from her consanguineous parents. This mutation creates an alteration of the splicing donor site on the last adenosine of the exon 5, changing an alanine to a threonine. The expected transcript product therefore contains a part of intron 5, leading to a shortened protein due to the occurrence of a stop codon 30 amino acids later (Figure 2).

The pathological effect of these mutations was ascertained by functional analysis (Table 1) as the two cases exhibited a near complete loss of CTSC activity.

Conclusion

We describe two previously unreported point mutations, p.A253SfsX30 and p.G386R, and an intragenic deletion

spanning exons 3–7 that represents a previously unreported mutational mechanism in *CTSC*. We propose that such intragenic deletions should be searched for in patients with apparent homozygous mutation when one or both parents are not available for genetic analyses or did not carry the inherited heterozygous mutation as would be expected. In the latter situation, false paternity and parental isodisomy have to be excluded using microsatellite analyses before performing gene dosage analysis by QMF-PCR.

MATERIALS AND METHODS

Patients

Patient 1 was a 20-year-old Caucasian woman. At age 14 years, she presented with severe periodontitis and keratoderma. Three years later, periodontitis and hyperkeratosis worsened to complete teeth loss and multiple cutaneous abscesses occurred. The diagnosis of PLS was then proposed and confirmed by molecular analyses. Her parents were healthy.

Patient 2, a 5-year-old Moroccan girl whose parents were first cousins, presented with a palmoplantar keratoderma in the first weeks of life and abnormal decidual tooth loss at the age of 3 years, associated with severe periodontitis. The parents were healthy. Informed consent was obtained from patients or parents. This research followed the tenets of the Declaration of Helsinki Principles. Institutional approval was not required for experiments.

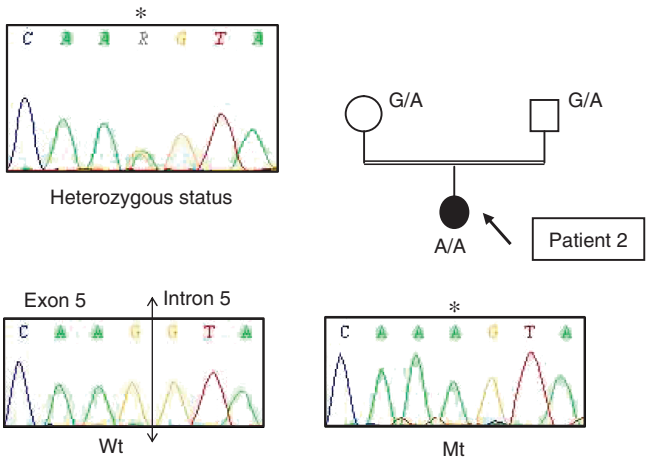


Figure 2. *CTSC* mutation analysis of patient 2. Patient 2, issued from consanguineous Moroccan parents, carried a homozygous c.757G>A mutation (p.A253SfsX30), inherited from both parents. This substitution causes a splice site mutation with the occurrence of a stop codon 30 amino acids downstream. Electrophoregrams show the homozygous wild-type sequence (Wt), homozygous-mutated sequence (Mt), and heterozygous status of both parents. The asterisk shows the location of the base transition.

Mutation analysis of CTSC

PCR and sequencing reaction. The *in vitro* amplification and sequencing of all *CTSC* exons and intron–exon boundaries were performed as described elsewhere (Toomes *et al.*, 1999; Lefèvre *et al.*, 2001). Mutations are described in accordance with the *CTSC* cDNA sequence GenBank NM_001814.2. The predicted effect is based on the protein sequence GenBank NP_001805.1.

QMF-PCR. The seven exons of *CTSC* were analyzed using a method adapted from Niel *et al.* (2004). Primers used for amplification are available from the authors upon request. In each set, two external controls were used: *DSCR1* exon 4 (located on chromosome 21) (Solassol *et al.*, 2003) and *coagulation factor IX* (*FIX*) exon 5 (located on chromosome X). Each fragment was amplified using one primer labeled with the fluorescent phosphoramidite 6-FAM dye (Sigma-Aldrich, L'Isle d'Abeau Chesnes, France). The PCR reactions were performed in duplicate in 25 μl reactions using the QIAGEN Multiplex PCR kit (Qiagen, Courtaboeuf, France), with 100 ng of genomic DNA and a mix of primers (concentration range from 0.2 to 0.6 mM). The reactions started with an initial denaturation step of 15 minutes at 95°C, followed by 23 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and a final extension of 7 minutes at 72°C. After electrophoresis on an ABI 3130XL sequencer (Applera, Courtaboeuf, France), data analysis was performed with the GeneMapper software (Applera). The results were first analyzed visually by superimposing fluorograms of patients and normal controls, normalization being performed with respect to

Table 1. Genetic and functional analyses of patients 1 and 2 and their first-degree relatives

Patients and relatives	Mutations	Predicted effect	Site	Type	Status	CTSC activity
1	c.1156G>C	p.G386R	Exon 7	Missense	ch	30 (<5%)
	Gene deletion exons 3–7	Truncated protein		Intragenic deletion		
Mother	c.1156G>C	p.G386R	Exon 7	Missense	hz	4680 (~80%)
Father	Gene deletion exons 3–7	Truncated protein		Intragenic deletion	hz	5880 (~100%)
2	c.757G>A	p.A253SfsX30	Exon 5	Altered splicing	hm	0 (<5%)
Mother					hz	290 (~20%)
Father					hz	395 (~27%)

ch, compound heterozygous mutation; CTSC, cathepsin C; hm, homozygous mutation; hz, heterozygous mutation.
CTSC-specific activity is expressed as nanomoles of NHMec produced per hour and per milligram of cell protein. In brackets, the activity is expressed as percent of the control values determined in the same experiment. CTSC activity was determined at least in duplicate in control samples, and in quadruplicate in patients' samples.

values obtained with control locus *DSCR1*. The peak height values were also imported into a custom Excel spreadsheet, and the copy number of each fragment was determined by calculating a fluorescence ratio for each exon relative to all the other amplified exons in patients and controls. Theoretical ratios of 1 and 0.5 are expected for a normal sample and a heterozygous deletion, respectively.

Functional analysis of CTSC

CTSC activity was determined by measuring the amount of NHMec released by hydrolysis of the synthetic substrate glycyl-L-arginine-7-amino-4-methylcoumarin (H-gly-arg-NHMec, Bachem, Merseyside, England) on incubation with sonicated peripheral blood leukocytes, using the modified protocol of Toomes *et al.* (1999). Each assay included controls in which either substrate or sonicated cells were omitted from the reaction mixture.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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